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COMMENTARY

Human biomonitoring and the ^{32}P-postlabeling assay

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Introduction

Biomonitoring and biomarkers

The routine monitoring of humans exposed to the plethora of anthropogenic genotoxic chemicals prevalent in the daily environment seems a formidable task. Epidemiological as well as experimental studies indicate that exposure to the majority of these chemicals is attributable to environmental, cultural and/or socioeconomic determinants (1,2).

Classical monitoring studies estimated exposure to genotoxic agents based on the levels of compounds present in ambient environmental media (3). The utility and relevance of this approach to human exposure and risk assessment studies has obvious limitations. Estimates based on external exposure predict only the approximate dose received by an organism and give no information regarding interindividual differences in absorption, metabolism, excretion, bioavailability, etc. (3).

A generally more indicative and integrated means of estimating and evaluating an individual's exposure is through biological monitoring. Biological monitoring provides information concerning: (i) the internal dose, a measure of parent chemical and/or metabolite in a body compartment or fluid; or (ii) the critical dose that actually gets to the site of action or interacts with critical cell macromolecules such as DNA (3,4). Biomonitoring coupled to the use of biomarkers adds a quantitative aspect to epidemiological studies, which are otherwise compromised by a lack of this integral component (4). Biomarkers of exposure include measurable biochemical, physiological, cytological, immunological or molecular changes in a biological system related directly or indirectly to exposure as well as measurable levels of metabolites in body fluids or compartments (5–11).

Biomarkers of carcinogen exposure

Molecular epidemiologists seek to elucidate the relationship(s) between exposure to a chemical(s) and the continuum of events following exposure (Figure 1) that potentially result in adverse sequelae (5,7). Ultimately, knowledge of the relationships between various stages in the continuum permit biomarkers taken at any stage to provide information relevant to other stages (5,12). This would be of obvious utility in the risk assessment of human cancer based on early exposure data owing to the extreme latency of the multifactorial human cancer process (4).

In the assessment of carcinogen exposure, biomarkers are chosen based on a knowledge of the internal interactions of carcinogen molecules/metabolites with cellular macromolecules such as DNA. While various chemical dosimeters for assessing exposure to genotoxic chemicals exist, methodologies for evaluating exposure involve essentially two approaches: (i) direct measurement of carcinogens and their metabolites in body fluids or excreta such as serum (13–15), breast milk (16,17) and urine (18,19); or (ii) measurement of covalent binding (adducts) between carcinogens or mutagens and DNA, RNA or proteins (13,20).

The utilization of body fluids to assess exposure has been widely applied to both animal and human studies. However, due to the rapid clearance of many chemicals from the body, this approach has assessment utility for only a short time post-exposure (5). Assessment of exposure weeks to months following actual exposure requires more stable markers such as adducts. DNA adducts have differing stabilities (hours–months) due to DNA repair, chemical instability, processes of cell turnover, etc, while protein adducts, if chemically stable, persist for the lifetime of the protein (5). Urinary excretion of DNA adducts removed from the genome by depurination/DNA repair mechanisms has also been measured as a biomarker for exposure assessment.

Protein adducts

Protein adducts, though not discussed in detail here, are considered important surrogate dosimeters for DNA adducts in monitoring carcinogen exposure. Amino acid residues such as histidine, cysteine and *N*-terminal valine in proteins interact with carcinogen metabolites. Although proteins are not considered to be critical targets for genotoxicants *per se*, it is their abundant accessibility from human blood in the form of hemoglobin and serum albumin that has prompted their use in biomonitoring (21).

DNA adducts as dosimeters of carcinogen exposure

Carcinogens, notably polycyclic aromatic hydrocarbons (PAHs*), are metabolically activated to electrophilic species that bind covalently to nucleophilic sites in DNA and form adducts (22). DNA adducts may occur at a number of sites within the DNA molecule; however, adduct formation involves specific electronic and stereochemical factors such that binding, especially with bulky aromatics, is not simply random (23,24). Guanine bases in DNA are the predominant sites for attack by chemical carcinogens. Which position of guanine a carcinogen would interact with is dictated by its chemical nature. For instance, the N7 position of guanine is predominantly modified by methylating or ethylating agents, while aromatic amines such as 4-aminobiphenyl and PAHs such as benzo[*a*]pyrene (B[*a*]P) prefer the C8 and N^2 positions, respectively (Figure 2) (3). In addition, other sites on guanine and sites on other bases may become

*Abbreviations: PAH, polycyclic aromatic hydrocarbon; B[*a*]P, benzo[*a*]pyrene; IQ, imidazoquinoline; IQx, imidazoquinoxaline; PhIP, phenylimidazopyridine; WBC, white blood cell; PBL, peripheral blood lymphocyte; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; USERIA, ultrasensitive enzymatic radioimmunoassay; BPDE, 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; SFS, synchronous fluorescence spectroscopy; MN, micrococcal nuclease; SPD, spleen phosphodiesterase; E:S, enzyme/substrate ratio; LFU, lithium formate and high molarity area, pH 3.5; LTU, lithium chloride, Tris and high molarity area, pH 8.0; DRZ, diagonal radioactive zone; RAL, relative adduct labeling; AHH, aryl hydrocarbon hydroxylase; RWC, residential wood combustion smoke; FAP, familial adenomatous polyposis; NNK, 4-[methylnitrosamino-1(3-pyridyl)]-1-butanone; DRM, DNA-reactive metabolites.

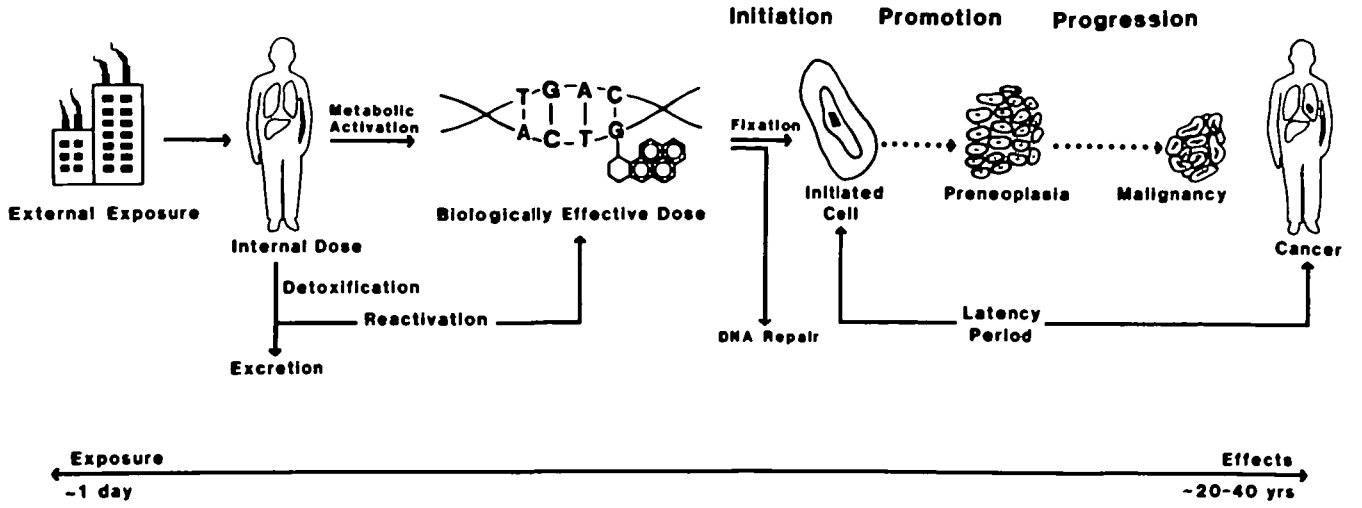

Fig. 1. Continuum of events in the multistage process of cancer.

significantly adducted as well, indicating the multiplicity of adducts from a single carcinogen. Although adducts are related to exposure, it appears that the extent of DNA damage occurring at specific sites is more important in terms of carcinogenicity. DNA adducts, if not repaired or misrepaired prior to cell replication, can induce gene mutation and putatively 'initiate' the exposed cell(s) converting it to an irreversibly altered preneoplastic lesion (25,26). Once a cell is initiated it is hypothesized to undergo subsequent stages known as promotion and progression (Figure 1) on its way to potential malignancy (26). Therefore, DNA adducts represent an early, detectable and critical step in the chemical carcinogenesis process, and thus may serve as an internal dosimeter of carcinogen exposure (5,27,28). Experimental evidence provided by mouse skin tumorigenicity studies suggests that further adduct-forming events, late in the cancer process, may also enhance the conversion of benign papillomas into malignant squamous cell carcinomas (29,30).

A causal and quantitative relationship between adduct formation and carcinogenicity has indirectly been suggested by the following: (i) adduct-forming capacity and *in vivo* carcinogenic potency correlate for many PAHs and alkylating agents (31–33); (ii) adduct formation and *in vitro* cell transformation/tumor induction have a positive correlation (34); and (iii) adducts are generally higher in the target organs of sensitive versus resistant animal species (35).

DNA damage in the form of adducts has been shown to activate oncogenes (36–38) and is speculated to affect expression of regulatory and tumor suppressor genes (39). Aromatic amines have been implicated in base transversions of the type known to activate the *ras* family of oncogenes (codons 12 or 61) (40). PAHs such as B[*a*]P have been shown to induce G → T transversions in the 12th codon of the *ras* protooncogene while 7,12-dimethylbenz[*a*]anthracene causes A → T transversions in the 61st codon, both of which may result in point mutation and conversion of the gene to an active oncogenic form (41). Individuals occupationally exposed to PAHs as well as smokers and lung cancer patients have been shown to contain elevated levels of the *fes* and *ras* oncogene protein products in their blood serum (42,43).

DNA adducts produced in human tissues/cells *in vitro* are qualitatively similar to those found in animals exposed *in vivo*, supporting extrapolation of animal bioassay data to the human

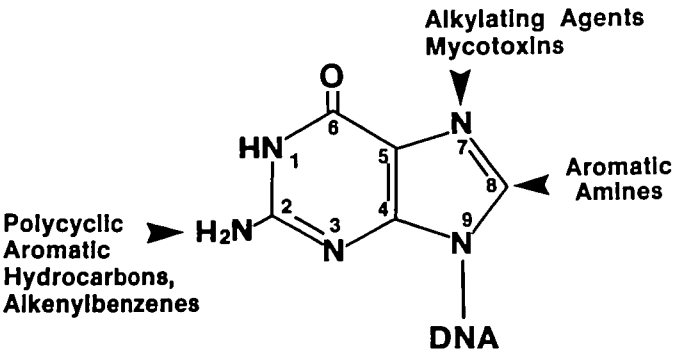

Fig. 2. DNA 'adduct library' indicating the preferential binding site on guanine for several classes of chemicals.

scenario and the use of DNA adducts as dosimeters of human exposure to genotoxic agents (13,44–46).

The 'human situation'

The assessment of human exposure to carcinogenic/mutagenic agents is a complex problem previously limited to epidemiological studies. Animal models have led to the development of the current multistage theories of carcinogenesis, and thus have strongly influenced the development of techniques for assessment of carcinogen exposure. The human situation, however, is much more multivariate compared with typical animal studies. There are at least three major factors, inherently unique to human exposure/cancer risk assessment, that dictate the applicability of approaches for their detection: (i) large interindividual variation; (ii) exposure to complex mixtures and confounding factors; and (iii) inaccessibility of human tissues suitable for analysis.

Many chemicals are metabolically activated by oxidative enzymes, such as cytochrome P450, to various electrophilic and carcinogenic metabolites. A number of P450 isozymes have been identified in humans with >1000-fold interindividual differences in terms of quantity, substrate specificity and tissue distribution (47–51).

Predisposition to cancer has also been correlated to genetic polymorphisms of non-inducible hepatic aromatic amine *N*-acetyltransferase (52). Phenotypically 'slow-acetylators' have been shown to have elevated risk for urinary bladder cancer and to form higher levels of adducts (hemoglobins) versus 'rapid-

acetylators' (52,53). Rapid-acetylation phenotype, however, has been shown to increase risk for aromatic amine-induced colorectal cancers (52).

DNA repair enzymes, responsible for the removal of adducts, may also play a role in genetic predisposition to cancer. Depressed rates of DNA repair have been observed in patients with xeroderma pigmentosum, a disorder that increases susceptibility to UV-induced skin cancer (54). O^6-Alkyl-DNA alkyltransferase, which repairs alkylated O^6-dG residues, has also been shown to be decreased in fibroblasts of patients with lung cancer versus non-cancer controls (54).

Broad interindividual variation in metabolism or adduct repair may explain some of the observed differences in human cancer risk. Cancer development and DNA adduct formation has been shown to be elevated in animals with increased capacity for the activation of procarcinogenic compounds (35).

Humans, in addition to their genetic variability, are chronically exposed to low doses of complex chemical mixtures. Contained in these mixtures are many known or potential carcinogens. One may encounter multiple sources of exposure in daily life (Figure 3). In addition to exposure via occupation, juxtaposition to environmental/industrial pollution (air, water) and UV radiation, lifestyle is also an important factor when considering the total scheme of exposure events. Cigarette smoking, environmental tobacco smoke, exhaust byproducts, foods, etc., may all contribute to a person's cumulative risk for cancer. A brief summary of these complex mixtures is presented.

Epidemiological observations as well as experimental animal studies have provided irrefutable evidence of the link between human lung cancer and tobacco smoke (reviewed in 55). Of the more than 3000 chemicals present in cigarette smoke (56), >50, including PAHs, aromatic amines, heterocyclic amines and *N*-nitrosamines, have been demonstrated to be either complete carcinogens, initiators or promoters in various *in vitro* or animal bioassays (57–59). Smokers have been shown to contain DNA adducts at levels greater than non-smokers with a dose–response relationship between consumption and adduct level (60). Smoke-related adducts have also been shown in experimental animals (61,62).

One of the most interesting and truly controversial areas of study concerns exposure to environmental tobacco smoke or passive/sidestream smoking. Several studies associating passive smoke and lung cancer have appeared since 1981 (63). Passive smoking is likely a major confounding factor which contributes to the 'background' DNA damage in humans typically assessed as controls.

Air pollutants other than cigarette smoke have been implicated in cancer causation in urban populations (64). Much of the data implicate PAHs and nitro-PAHs produced during the pyrolysis of fossil fuels by industry and transportation (diesel and gasoline exhaust). Coke oven plants and aluminum refineries have also been shown to emit substantial levels of PAHs into ambient air (65). Drinking water, like air, is an essentially unavoidable complex mixture to which we are inevitably exposed. Many mutagens and carcinogens, including trihalomethane, have been found in chlorinated drinking water (66,67). A chlorinated furanone [3-chloro-4(dichloromethyl)-5-hydroxy-2(5*H*)-furanone] accounts for up to half of the Ames mutagenicity of drinking water (66,68). Groundwater has also been shown to be contaminated in many areas with a variety of organics, pesticides and metals (66).

Food constituents may be one of the most important complex mixtures to which humans are exposed. Carcinogens found in food include mycotoxins, plant alkaloids, *N*-nitroso compounds, PAHs, nitroarenes and heterocyclic amines (69). Extensive work has been done on the formation of heterocyclic amines as a result of the pyrolysis of meat products. Compounds include imidazoquinoline (IQ), imidazoquinoxaline (IQx) and phenylimidazopyridine (PhIP). PhIP is known to produce DNA adducts in rat (70) and IQ in the livers of rats as well as primates (71). Like air and water, the contribution of food mutagens and carcinogens may confound the interpretation of cancer risk assessment.

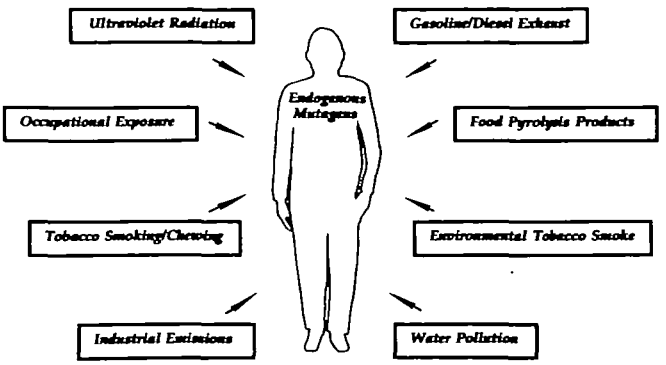

Fig. 3. Indication of multiple sources of exposure to complex mixtures of carcinogens/mutagens potentially encountered in the 'daily life' of a human.

Finally, a major difficulty in assessment of human exposure to carcinogens is the inaccessibility of suitable tissues for DNA adduct analysis. Many studies have utilized white blood cells (WBCs) or peripheral blood lymphocytes (PBLs) as surrogate tissues, while others have analyzed samples from autopsy tissue, placental tissues or limited numbers of biopsy samples (72). Further animal studies are necessary to establish the relationship of surrogate tissue to target tissue and hence their potential for exposure assessment.

The development of appropriate methodologies for detecting and quantifying DNA adducts as biomarkers is dictated by the human scenario described above. For an assay to possess potential as a screening device for exposure it must: (i) be sensitive enough to detect the interaction of chemicals with DNA at low (environmental) levels; (ii) detect adducts from human samples accessible on a routine basis which provide little DNA; (iii) be quantitatively related to exposure; (iv) be applicable to detection of adducts produced by complex mixtures of chemicals including unknowns; and (v) be able to resolve, quantitate and identify adducts.

DNA adduct detection methodologies

Extremely sensitive and highly sophisticated methods for assessing and quantifying DNA adducts have been developed over the last decade. Three distinct approaches have been in use and will be discussed briefly: (i) immunoassays; (ii) fluorescence assays; and (iii) ^{32}P-postlabeling. All three methodologies have advantages and disadvantages which delegate their place in the scheme of human exposure assessment. These methods represent marked technological improvements in the field of adduct assessment and have extended the detection limits for carcinogen–DNA adducts to 1 per 10^6–10^{10} normal nucleotides; within human applicability.

Immunological techniques have been employed for adduct measurement including competitive radioimmunoassay (RIA), as well as solid phase, competitive or non-competitive enzyme-linked immunosorbent assay (ELISA) or ultrasensitive enzymatic

Table I. Individual agents or complex mixtures tested by ^{32}P-postlabeling (total = 141)

Alkylating agents
Dacarbazine
Diethyl sulfate
1,2-Dimethylhydrazine
N,N-Dimethylnitrosamine
Dimethyl sulfate
Ethylene oxide
Ethylnitrosourea
N-Methyl-*N*-nitrosourea
Procarbazine
Propylene oxide

PAHs
Benz[*a*]anthracene
Benzo[*a*]fluorene
Benzo[*b*]fluorene
Benzo[*b*]fluoranthene
Benzo[*c*]fluoranthene
Benzo[*j*]fluoranthene
Benzo[*k*]fluoranthene
Benzo[*g,h,i*]perylene
Benzo[*a*]pyrene
Benzo[*e*]pyrene
Chrysene
Dibenz[*a,c*]anthracene
Dibenz[*a,h*]anthracene
Dibenzo[*a,e,l*]fluoranthene
Dibenzo[*a,e,l*]pyrene
Dibenzo[*a,i*]pyrene
Dibenzo[*a,l*]pyrene
7,12-Dimethylbenz[*a*]anthracene
Fluoranthene
Indeno-[1,2,3-*c,d*]pyrene
3-Methylcholanthrene
Triphenylene

Heterocyclic PAHs
Benzo[*f*]quinoline
Dibenzo[*a*]acridine
Dibenzo[*a,i*]carbazole
Dibenzo[*c,g*]carbazole
Quinoline

Nitro-PAHs
Aristolochic acid I
Aristolochic acid II
1,8-Dinitropyrene
2-Nitrofluorene
1-Nitropyrene
4-Nitroquinoline-1-oxide

Cyclopenta-fused PAHs
Aceanthrylene
Acenaphthylene
Acephenanthrylene
Benz[*j*]aceanthrylene
Benz[*l*]aceanthrylene
Cyclopenta[*d,e,f*]phenanthrene
Cyclopenta[*c,d*]pyrene

Aromatic amines
2-Acetylaminofluorene
4-Acetylaminofluorene
2-Acetylaminophenanthrene
4-*trans*-Acetylaminostilbene
4-Aminobiphenyl
1-Aminopyrene
2-Anthramine
Benzidine
4-Dimethylaminoazobenzene
4-Methylaminoazobenzene
4,4′-Methylene-*bis*(2-chloroaniline)
2-Naphthylamine
2,4-Dinitrotoluene
2,6-Dinitrotoluene

Dyes
Congo Red
Evans Blue
HC Blue I
Sudan I

Alkenylbenzenes
Allylbenzene
Anethole
Dill apiol
Elemicin
Estragole
Isosafrole
Methyleugenol
Myristicin
Parsley apiol
Safrole

Quinones
Benzoquinone
Ellagic acid
Hydroquinone
Lucidine

Mycotoxins
Aflatoxin B_1
Sterigmatocystin

Chemotherapeutic agents
1,2-*bis*(2-Chloroethyl)-1-nitrosourea
Cyclophosphamide
Diaziquone
Mitomycin C
Nitrogen mustard
cis-Platin
Streptozotocin

Chemicals producing cyclic adducts
Acrolein
Crotonaldehyde
Ethyl carbamate
Glycidaldehyde
Vinyl carbamate
Vinyl chloride

Pesticides
Dichloflaunid

Aldehydes
Formaldehyde

Complex mixtures
Cigarette mainstream smoke
Cigarette sidestream smoke
Chewing tobacco/sniff dipping
Coal soot
Coal-tar
Coke oven emission
Crude oil
Diesel exhaust
Diesel-2 oil
Juniper tar
Petroleum oil
Residential wood smoke

Aminoimidazoazaarenes (food mutagens)

AαC	2-amino-9*H*-pyrido[2,3-*b*]indole
4,8-DiMeIQx	2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
7,8-DiMeIQx	2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline
Glu-P-1	2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole
Glu-P-2	2-aminodipyrido[1,2-*a*:3″,2″-*d*]imidazole
IQ	2-amino-3-methylimidazo[4,5-*f*]quinoline
MeAαC	2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole
MeIQ	2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline
MeIQx	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
Trp-P-1	3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole
Trp-P-2	3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole

Radiation/radical damage
Hydrogen peroxide
Potassium permanganate
γ-Radiation
UV light

I-compound modulators
Carbon tetrachloride
Ciprofibrate
Diethylstilbesterol
Estradiol
Estrogen
Hexestrol
11β-methyl-17α-ethinylsestradiol
2,3,7,8-Tetrachloro-*p*-dibenzodioxin
1,2,3,7,8-Pentachlorodibenzodioxin
Wy-14643

Others
2,2′,4,4′,5,5′-Hexachlorobiphenyl
β-Propiolactone
Pentabromobiphenyl
Styrene

radioimmunoassay (USERIA) (55). These assays are typically sensitive enough to detect 1 adduct per 10^8 nucleotides using 25–50 μg DNA (72). A major limitation of these assays is cross-reactivity. Antibodies against 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE)-modified DNA have been shown to cross-react with other PAH adducts including chrysene and benz[*a*]anthracene (72). A further limitation to this assay is its requirement for a new antibody for every adduct to be detected and an inability to detect adducts from unknown chemicals.

An alternative approach to adduct detection utilizes the inherent fluorescent properties of many PAHs. Direct measure of aflatoxin B_1 and some PAH adducts has been made by fluorescence in cases where binding levels were quite high and 100 μg of DNA were available (73). The most widely applied approach is scanning synchronous fluorescence spectroscopy (SFS) where simplified spectra are generated by simultaneous scanning of excitation and emission wavelengths at a fixed wavelength difference (74). Sensitivity is reported between 3 and 10 adducts per 10^8 nucleotides. Disadvantages to the approach include lack of quantitation due to broad peaks in many samples studied, cross-detection of other unrelated fluorophores and an inability to screen human samples for non-fluorescent compounds and unknowns derived from complex mixtures. An advantage for this assay

would be its use as a complimentary validation assay alongside the immunoassays. A newer and potentially more sensitive fluorescence-based assay is fluorescence-line narrowing (75). Laser excitation of BPDE-modified DNA cooled to 4.2K dramatically narrows fluorescence spectra (75). Current sensitivity is 1 per 10^6 but is likely to improve.

The ^{32}P-postlabeling method, discussed in detail below, is ideally suited to the biomonitoring of humans. It can be used to detect adducts in the 1 per 10^7-10^{10} range using $1-10$ μg of DNA. It is not chemical specific, theoretically allowing for detection of any chromatographically separable adduct (Table I). Thus, it can detect unknowns derived from the complex mixtures to which humans are exposed.

This commentary was thus far designed to discuss briefly the concepts of human biomonitoring/biomarkers, DNA adducts as suitable biomarkers of exposure to carcinogenic agents, the complexity of the human situation in terms of exposure assessment and available technology appropriate for screening and assessing human DNA adducts. The following sections cover aspects of each step in the ^{32}P-postlabeling assay, potential problems, advantages, limitations, human studies so far completed and future directions for ^{32}P-postlabeling and human biomarkers in general.

The ^{32}P-postlabeling assay

The ^{32}P-postlabeling assay is most noted for its sensitivity (1 adduct per 10^{10} nucleotides) and applicability to structurally diverse classes of chemicals (Table I). The basic assay (Figure 4) involves a stepwise sequence of biochemical reactions entailing: (i) cleavage of intact DNA to the deoxynucleoside 3′-monophosphate level; (ii) enrichment of adducted nucleotides by elimination of normal nucleotides; (iii) attachment of a ^{32}P label to the 5′-hydroxyl end of adducted nucleotides creating a 3′,5′-bisphosphate; (iv) separation and detection of adducts by high-resolution TLC and autoradiography respectively; and (v) quantitation of adducts by measurement of radioactivity.

Steps (i)–(iii) involve the time-dependent incubation of DNA/nucleotides with various enzymes and their cofactors. Step (iv) involves the use of anion-exchange, PEI–cellulose TLC plates either made in the laboratory or purchased from commercial sources. Adducts are separated in step (iv) by variable multi-directional solvent schemes. Each of these steps is subject to modification by the researcher, and therefore represents a point of variation that may contribute to losses of adducts and interlaboratory variation (76,77).

It should be noted that many changes (additions and deletions) have been made in this assay since its appearance in the literature in 1981/1982. Several versions of the assay exist including (i) standard assay (78,79); (ii) intensification (ATP-deficient) approach (80); (iii) butanol enrichment (81); (iv) nuclease P_1/S_1 enrichment (82,83); (v) the dinucleotide/nucleoside 5′-monophosphate assay (84); and (vi) the HPLC enrichment assay (85). The standard and ATP-deficient versions of the assay lack an enrichment step. The latter procedure enhances the detection limit for some adducts due to enzymatic preference (kinase labels certain adducts in preference to normal nucleotides when carrier-free ATP is used and it is limiting). In both procedures sensitivity is usually in the range of 1 adduct per 10^7-10^8 nucleotides, due to either a limited amount of DNA (≤ 0.2 μg in the standard assay) or incomplete labeling (ATP-deficient version). Labeling of adducts after their enrichment with either butanol, nuclease P_1/S_1 or HPLC allows for larger amounts of DNA (usually

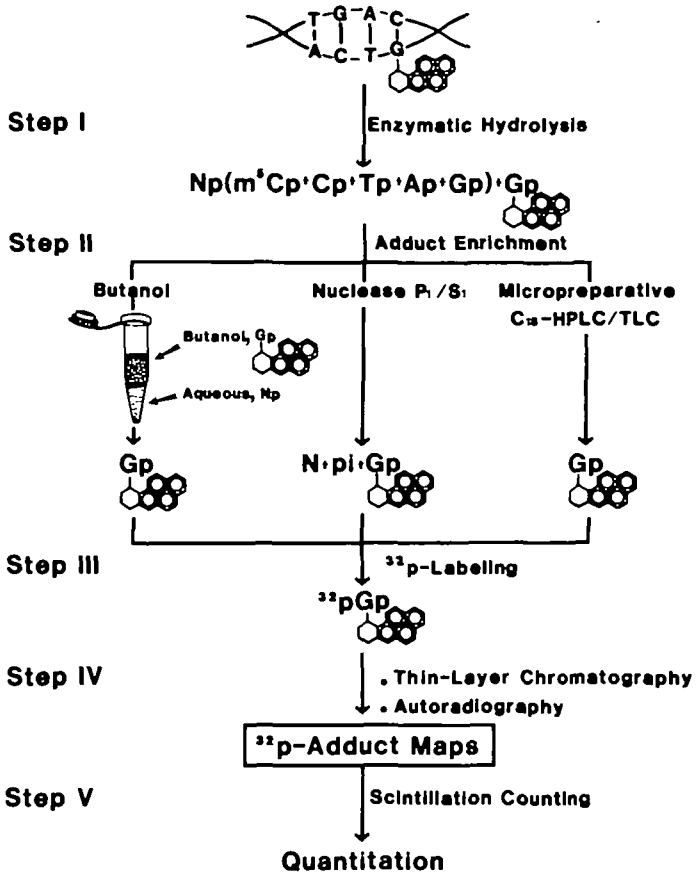

Fig. 4. Representation of various adduct enrichment versions of the ^{32}P-postlabeling assay.

$5-10$ μg) and a molar excess of carrier-free ATP to be used (as normals are virtually absent during labeling) increasing sensitivity to 1 adduct per 10^{10} nucleotides. The dinucleotide/nucleoside 5′-monophosphate assay is a somewhat different approach versus the other versions of the assay. DNA is initially cleaved by nuclease P_1 to yield 5′-monophosphates of normal mononucleosides (pN) and in theory 5′-phosphate dinucleotides containing an adducted base 5′ to a normal base (pXpN). Prostatic acid phosphatase is added to 5′-dephosphorylate both the normal mononucleotides (pN → N) and adducted dinucleotides (pXpN → XpN). Dinucleotides are then labeled (^{32}pXpN) and can be mapped or further digested to labeled mononucleotide 5′-monophosphates (^{32}pXpN → ^{32}pX) via snake venom phosphodiesterase and then mapped (84).

Adduct enrichment with HPLC prior to ^{32}P-postlabeling has also been described (85). Digested DNA samples containing a mixture of normal (Np) and adducted (Xp) nucleotides are injected into a reverse-phase HPLC column. Normal nucleotides elute in 5% methanol/95% 1 M ammonium formate, pH 3.5. Adducted bases are recovered with either a linear methanol gradient to elute adducts in separate fractions or a step gradient to elute all adducts in a single fraction (85). Recovered fractions are dried, postlabeled with ^{32}P and resolved by PEI–cellulose TLC. HPLC enrichment provides qualitatively comparable results to nuclease P_1 enrichment; however, background is typically higher and adduct recovery lower (20–50%) (85). Advantages to HPLC are its ability to fractionate adducts for individual mapping on TLC layers and its recovery, like butanol, of adducts

labile to nuclease P_1-catalyzed 3′-dephosphorylation. HPLC enrichment has been used to detect lipophilic adducts in fish from polluted versus non-polluted waterways (86,87) and human bronchial biopsies (88).

The standard ATP-deficient assays were used largely before enrichment techniques were available (butanol, 1985; nuclease P_1, 1986; HPLC, 1988). They are now utilized only in cases where adducts cannot be sufficiently enriched. The ATP-deficient procedure has been largely employed in the recovery of adducts induced by the heterocyclic amines found in cooked foods (89–91). The dinucleotide assay has also been of limited use and would seem to be applicable only in cases where adducts preclude complete DNA digestion via the bisphosphate assay, and thus complete recovery of adducts. HPLC enrichment has also, thus far, seen limited use. The nuclease P_1 and butanol versions of the assay have been extensively applied to the human situation, and will be the subject of further comments.

The purpose of this section of the paper is to point out the inherent objectives at each stage of the postlabeling assay. Included here are comments regarding the approaches used in different laboratories and mention of what is known or considered to be optimal. It should be stressed that conditions need to be selected based on the adduct or class of adducts being analyzed.

Pre-assay measures—isolation of DNA

Prior to initiating the postlabeling assay it is of vital importance that high quality, RNA/protein-free DNA is prepared. Many laboratories appear to follow the original modified, solvent extraction procedure described in 1984 (24). Tissue is homogenized and treated with proteinase K (500 μg/ml, 37°C, 30 min) in 1% SDS/1 mM EDTA, pH 7.4, then extracted with 1 vol each of phenol, a 1:1 mixture of phenol/sevag (chloroform/isoamyl alcohol, 24:1) and sevag. DNA present in the aqueous phase is precipitated with 1 vol of chilled ethanol and 0.5 M NaCl, collected by centrifugation, and washed with 70% ethanol. Residual RNA is then removed by digestion with a mixture of RNases T_1 (50 U/ml) and A (100 μg/ml) and extractions with sevag. DNA is again precipitated with ethanol and finally dissolved in 0.01 × 0.15 M NaCl/0.015 M sodium citrate (SSC). Spectrophotometric estimation is based on the relationship of 20 A_{260} = 1 mg DNA. $A_{230/260}$ and $A_{260/280}$ ratios provide an indication of contamination with proteins and RNA respectively. RNA content can further be assessed and quantitated by one- or two-directional TLC during normal nucleotide analysis; RNA-derived nucleotides migrate somewhat slower than their DNA counterparts. The purity of DNA preparations can also be assessed by HPLC analysis (92). A contamination level of as little as 1% can be estimated using this procedure (Dr J.Ross, personal communication).

Modifications have been made since this procedure was first described. A change that increased the speed of this process was made where tissue ground in SDS/EDTA was first treated with RNases A and T_1, followed by proteinase K, the three solvent extractions and precipitation with ethanol. Thus, one set of solvent extractions is eliminated (93). We have also extensively modified the original procedure. Tissue is homogenized in 50 mM Tris–HCl/10 mm EDTA, pH 8.0, and crude nuclei are isolated using a table-top centrifuge (3000 r.p.m./10 min). Nuclei are treated with RNases A and T_1 (150 μg/ml and 20 U/ml respectively) in the presence of 50 mM Tris–HCl, pH 8.0, for 30 min, 37°C. This is followed by incubation with proteinase K, the three extraction steps, and precipitation in ethanol. We find that increasing the pH to 8.0 increases the DNA yield (in some cases substantially) and provides extremely high quality (RNA-free) DNA from both animal and human sources with no affect on adduct integrity (R.C.Gupta, unpublished results). The commonly used pH of pH 7.4 [pH 7.0 in some studies (94)] during tissue homogenization and solvent extractions can increase the potential for RNA contamination and decreases DNA yields. Methods that increase DNA yields are important in human studies considering the limited tissue samples available. RNA contamination is also possible in cases where RNase incubation is subsequent to proteinase K with no solvent extraction in between (95).

Co-precipitation of RNA and/or other contaminants is a major concern for the sensitive postlabeling assay. RNA nucleotides are substrates for all of the postlabeling reactions and may lead to 'false positives' (next section). Additionally, the precipitation of agents that affect kinase labeling have been found (Dr F.F.Kadlubar, personal communication). These 'unknown' contaminants could potentially affect any stage of the assay with enzymatic steps being likely targets. We are currently using commercially tested, HPLC-grade water for all assay reagents, etc., in an attempt to avoid water-borne contaminants, which have been observed to affect kinase labeling. This is especially important when using nuclease P_1 where aqueous contaminants would remain throughout the assay.

Step 1: enzymatic hydrolysis

The initial step in the postlabeling assay involves the hydroloysis of DNA to the 3′-nucleoside monophosphate level using a mixture of micrococcal nuclease (MN) and spleen phosphodiesterase (SPD). The objective of this step is to hydrolyze completely the DNA to mononucleotides under the least vigorous conditions without affecting adduct yield. Variations of this step have been reported in the literature with respect to both the amount of enzyme/μg of DNA and the enzymatic incubation time. The original assay detailed a reaction cocktail of 2 μg MN and 2 μg SPD/1 μg of DNA in 10 mM $CaCl_2$ and 20 mM sodium succinate, pH 6.0, 38°C, 2 h (79). We as well as others have since found that an enzyme to substrate ratio (w/w) (E:S) as low as 1:3, or currently 1:7, in 5 mM $CaCl_2$ and 10 mM sodium succinate, pH 6.0, is quite sufficient to hydrolyze DNA completely within 4–6 h at 37°C and increases the yield of certain adducts (81; also unpublished results). However, some laboratories still follow more vigorous digestion protocols with ≥5 times the needed quantity of MN/SPD (85,96–98) and lengthy incubation times of 16–24 h (96,99). Such lengthy incubations are likely to result in the loss of adducts. Increasing incubation to 14 and 20 h versus 4 h decreased the adduct level of dibenzo[*a*,*e*]fluoranthene 35 and 54% respectively (100). Aromatic amine adducts are particularly sensitive during MN/SPD digestion and can depurinate during long incubations (M.Chacko and RC.Gupta, unpublished data). A variation in which MN and SPD are incubated separately at their respective pH optima to increase the speed of the reactions has also been reported (101,102). The use of a mixture of the two enzymes at a suitable pH of 6.0 provides adequate function for post-labeling purposes and is therefore easier to perform. A digest performed at pH 8.8 for 2 h followed by a 10 min neutralization in 0.1 N HCl at 95°C has also been described (102). Such conditions should only be used under close scrutiny to ensure adduct stability/recovery.

Incubation time and MN/SPD concentrations used for a given purpose should be (and in many cases are) worked out by individual laboratories to ensure optimal adduct recovery.

Step 2: adduct enrichment

Possibly the most crucial but often overlooked phase of the assay is step 2, or adduct enrichment. The two most commonly used approaches to this step are different with respect to their working nature and the results they provide. Butanol enrichment involves the preferential partitioning of aromatic and/or hydrophobic adducts into this organic solvent over an aqueous phase (81). Normal nucleotides, however, remain in the aqueous phase. Nuclease P_1 and more recently S_1 (82,83) enrichment procedures take advantage of the base-specific 3′-phosphatase activity of these enzymes. Normal 3′-nucleoside monophosphates (Np) are readily hydrolyzed to their corresponding nucleosides (N) by nuclease P_1/S_1. Many adducted nucleotides (Xp), by virtue of their structure, are resistant to this hydrolysis and fail to act as nuclease P_1/S_1 substrates. Nucleosides are not substrates for subsequent kinase labeling, and thus only adducts (Xp) become labeled (^{32}pXp) in the presence of polynucleotide kinase (PNK) and [γ-^{32}P]ATP (82). Both methods are applicable to small or large samples and increase the sensitivity of the assay to 1 adduct per 10^{10} nucleotides. However, they differ dramatically with respect to their recovery of certain adducts. Since use of S_1 was only recently reported, rigorous analysis of its adduct selectivity has not yet been made.

In general, PAH adducts show similar recovery in both procedures while some PAH adducts are better recovered by the nuclease P_1 assay. Extensive analysis of the adduct selectivity of butanol, nuclease P_1, T_4 PNK (a 3′-phosphatase at acid pHs) and the ATP-deficient procedure have previously been reported (103,104). The dG-N^2-B[*a*]P triol adduct resulting from the incubation of BPDE with DNA is recovered equally well by either nuclease P_1 or butanol (Figure 5) (103). However, a prominent *in vivo* adduct reported for B[*a*]P (105) is completely lost during butanol enrichment (A.C.Beach, G.Spencer and R.C.Gupta, unpublished data). Adducts of dibenz[*a*,*h*]anthracene diolepoxide-modified DNA are recovered only 19–77% in nuclease P_1 versus butanol while the adducts of aceanthrylene are recovered at levels 2-fold higher in nuclease P_1 versus butanol versions. Aromatic amine adducts, such as the dG-C8 derivatives of 4-aminobiphenyl and 4-acetylbenzidine are highly sensitive (lost 96 and 97%) to nuclease P_1 versus butanol (Figure 5). Therefore, unless a system is predefined (i.e. adducts are known and well characterized in nuclease P_1 and butanol) a stepwise approach to choosing a suitable enrichment system for continued studies is vital. Nuclease P_1 is much less time consuming compared to butanol; however, when dealing with the environmental mixtures of unknown composition to which humans are routinely exposed, it would be an oversight simply to choose only one or the other method.

In many cases nuclease P_1 has been used to characterize adducts of poorly defined human samples from cigarette smoke-exposed individuals (60,88,106–109), foundry workers (76, 110,111), coke oven workers (77), coal tar-treated psoriasis patients (112,113) and roofers (114), without additional comparisons with butanol. In cases where smoking-induced adducts are of interest, adduct-forming aromatic amines such as 4-aminobiphenyl (present in much greater amounts in sidestream versus mainstream cigarette smoke) would not be detected if only the nuclease P_1 version is applied. dG-C8 arylamine adducts are especially sensitive to nuclease P_1-catalyzed dephosphorylation. The dG-N^2-adduct produced by 2-aminophenanthrene is stable in the presence of nuclease P_1, while the dG-C8 adduct is almost completely lost (Figure 5). Such differences in the results of the two assays can in fact be exploited for adduct characterization.

Oral mucosal cells from smokers have been subjected to both nuclease P_1 and butanol (115). As is readily seen in Figure 6, major adducts are lost in the enzymatic procedure. Differences in human bladder DNA adducts from smokers as well as the excretions of DNA-reactive metabolites related to PhIP have also been reported (116,117). However, other studies show identical recovery with either procedure of DNA from animals or humans exposed to complex mixtures (118,119). Once this has been ascertained, then nuclease P_1 is preferable for its ease of use.

Variations within the methods for step 2 are subject to further comments. The butanol method as originally described in 1985 (81) is still performed with only slight modification. The objective of this step is to extract as much of the adducted nucleotides as possible while accomplishing complete elimination of normal nucleotides from the sample. Two extractions with *n*-butanol are still used to capture ~95+% of extractable adducts. A third

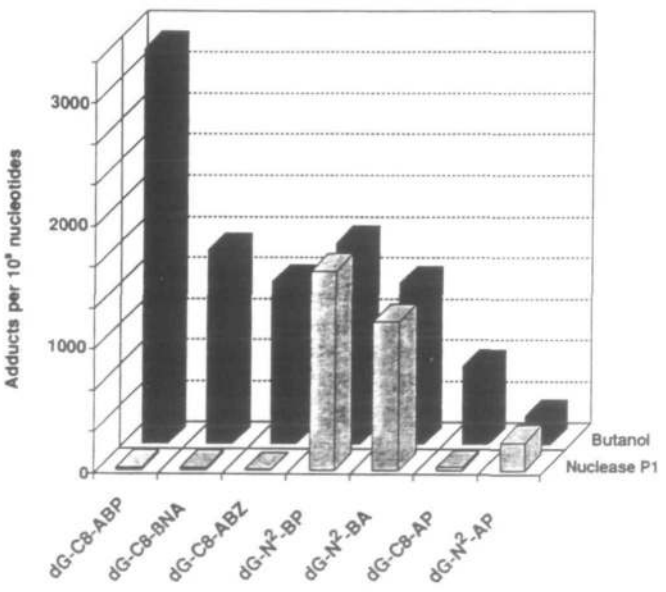

Fig. 5. Comparative adduct recoveries for various aromatic amine and PAH adducts in the butanol and nuclease P_1-enhanced versions of the ^{32}P-postlabeling assay (modified from data in ref. 103). ABP = 4-aminobiphenyl, βNA = β-naphthylamine, ABZ = acetylbenzidine, BP = benzo[*a*]pyrene, BA = benz[*a*]anthracene and AP = 2-aminophenanthrene.

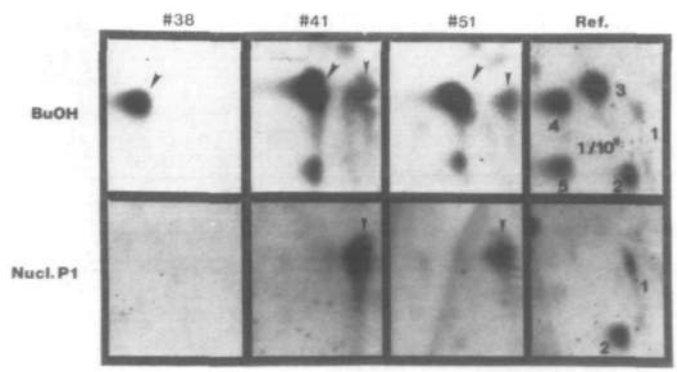

Fig. 6. Comparison of butanol and nuclease P_1 enhancement procedures for recovery of adducts from the oral mucosa of Indian tobacco chewers and a positive reference DNA; the numbers 38, 41, etc., represent specimen numbers (modified from data in ref. 115).

extraction does not add appreciably to the adduct yield yet significantly increases labor time. The water backwashes of the combined butanol fraction have recently been upscaled for larger samples to improve removal of normals prior to kinase labeling. For 5–30 μg DNA, three backwashes with butanol-saturated water are used, and for ≥30 μg four backwashes are needed (R.C.Gupta, K.Earley and M.Chacko, unpublished results). With these exceptions the assay remains essentially as published.

Nuclease P_1 enrichment protocols have been modified somewhat since originally described in 1986 (82). This enzyme is used to remove normal nucleotides so that they do not participate in subsequent labeling reactions. It is, therefore, important that the enzyme has minimal effect on adducts. As noted above and seen in Figure 5, many PAH adducts are recoverable with nuclease P_1 yet slightly labile. In this case, vigorous nuclease P_1 treatment may result in increased adduct loss. The original assay prescribed an E:S of 0.6:1 at 37°C for 40 min in the presence of 0.25 M sodium acetate, pH 5.0 (optimal for P_1), 0.3 mM $ZnCl_2$. We have found that a ratio (w/w) of nuclease P_1 to digested substrate of 1:5 and a pH of 6.0 (pH of the MN/SPD digest), in the presence of 0.1 mM $ZnCl_2$, at 37°C for 30 min, is still sufficient to eliminate normals and optimally recover adducts.

Other investigators have also cut down on the amount of nuclease P_1 used in their assay (from 1 U/4 μg DNA to 0.15 U/4 μg DNA) (113,119). Nuclease P_1 has been titrated to optimize adduct recovery from WBC-DNA of foundry workers (110). Digested DNA (4 μg) was enriched with nuclease P_1 using 0.625–10 μg/sample. Nuclease P_1 at E:S ≈ 1:1 decreased adduct recovery ~30% and E:S ≈ 2:1 resulted in ~50% losses. Adduct recovery for this study was optimal at E:S ≈ 1:4 (110). The variability of nuclease P_1/μg DNA in the literature ranges from about 0.3:1 to 6:1 (110,119–125), which is bound to contribute to interlaboratory variability (76,77).

We suggest a broad-based approach be taken when initiating the analysis of human samples from complex mixture exposures. Several investigators have used this approach and have compared butanol and nuclease P_1 in initial studies (102,115,117,124,126). Nuclease P_1 levels should also be titrated to ensure maximum recovery. It is strongly emphasized that the idea of this step is just to eliminate normal nucleotides (Np → N) without affecting potentially labile adducts, thus resulting in optimal yields of Xp for kinase labeling.

Step 3: ^{32}P-labeling

Radiolabeling following enrichment involves PNK-catalyzed enzymatic transfer of ^{32}Pi from [γ-^{32}P]ATP to the 5′-position of adducted nucleotides to create 3′,5′-bisphosphate nucleotides (Xp → ^{32}pXp). Crucial to this step is the presence of adequate PNK and a molar excess of [γ-^{32}P]ATP. The original butanol and nuclease P_1 enrichment protocols for labeling utilized 150–200 μCi [γ-^{32}P]ATP and 3–5 U PNK (81,82). Widespread variability in both the amount of PNK and [γ-^{32}P]ATP are reported within the literature. Additionally, many laboratories synthesize their own [γ-^{32}P]ATP by an enzymatic method described (79,81,82) or purchase it from commercial sources. High quality, carrier-free [γ-^{32}P]ATP with a sp. act. ≥3000 Ci/mmol should be used in either case. Specific activity can be assayed as described (81,82). If inadequate [γ-^{32}P]ATP is used, labeling will not proceed quantitatively. To ascertain that [γ-^{32}P]ATP is in excess, an aliquot (≤0.3 μl) of labeled solution can be spotted on a small piece of a PEI–cellulose layer and developed in 4.5 M ammonium formate, pH 3.5, as described (81) or 1.2 M lithium chloride. This 'transfer-efficiency' is a simple 5 min step ensuring that a molar excess of [γ-^{32}P]ATP was present. In our laboratory we find 100 μCi of [γ-^{32}P]ATP (~3000 Ci/mmol)/sample to be acceptable for aromatic adducts enriched (with either butanol or nuclease P_1) from 1 to 40 μg DNA. Many laboratories have determined that lower levels are sufficient for their purposes as published. Levels as low as 10 μCi (95,98,125) and 25–50 μCi (76,127–129) have been reported. However, any time that unknowns, such as those present in human samples, are assayed, a titration comparing [γ-^{32}P]ATP concentration and adduct recovery should be made and in all cases postlabeled solution must be analyzed to show that [γ-^{32}P]ATP was in excess. If labeling solution is even slightly contaminated with normal nucleotides, the use of very low levels of [γ-^{32}P]ATP is risky at best and may result in a lack of quantitative labeling. Further, levels as low as 10–20 μCi result in only ≤20% labeling efficiency; at least 50–60 μCi (~1–1.2 μM) is required to obtain plateau (our unpublished results). Increased levels of [γ-^{32}P]ATP (up to 10–60 μM) have been shown to be necessary to reach plateau for adducts such as thymidine glycols (130) and styrene oxide–DNA adducts (S.Cantoreggi, W.Lutz, U.Devanaboyina and R.C.Gupta, unpublished data). Some laboratories have used significantly higher levels of [γ-^{32}P]ATP, ranging from 200 to 380 μCi (107,116,117). Unless the necessity for these extreme conditions can be demonstrated, use of excessive [γ-^{32}P]ATP should be avoided to maintain minimal background noise.

The amount of PNK used and the pH of the labeling reaction are important variables in this step as well. In addition to phosphorylating activity at pH 8.0–9.5, PNK is also a 3′-phosphatase at somewhat acidic pHs (optimum ~ pH 6.0). Maintenance of high pH (~9.5) during labeling is therefore vital. However, excess of this enzyme may still promote the dephosphorylation of adducts. PNK is also supplied in 50% glycerol. The introduction of high levels of glycerol (via high concentrations of PNK) into the labeling mixture has been found occasionally to inhibit kinase labeling. PNK has been varied between 2 and 67.5 U (107,116,117,131). We routinely use 2–3 U PNK and find this sufficient for labeling adducts derived from 1–40 μg of DNA in the presence of 100 μCi [T-^{32}P]ATP (~2 μM) and the appropriate buffer, pH 9.5, in a total volume of 15 or 20 μl incubated for 40–45 min at room temperature (~22°C). However, other investigators have reported that the use of Boehringer Mannheim (Indianapolis, IA) phosphatase-free kinase at a pH of 7.2 provides labeling efficiency equivalent to that at pH 9.5 (Dr J.Ross, personal communication).

An additional step using potato apyrase (60 mU) to remove excess [γ-^{32}P]ATP prior to TLC was originally described and is still utilized to a large extent in recent studies. Apyrase is not necessary in experiments involving aromatic adducts and improved chromatography (see below). In addition, commercial apyrase is typically contaminated which may affect adduct recoveries.

Step 4: adduct separation and visualization

Thin-layer chromatography. Subsequent to adduct labeling, samples or aliquots of samples are spotted on PEI–cellulose layers for multidirectional TLC as described (79,81,132). Conditions for TLC may also be highly variable; however, many investigators still rely heavily on the original system described in 1982 (79) with the substitution of sodium phosphate (NaH_2PO_4) for lithium chloride in D1, the deletion of D2, and the addition of NaH_2PO_4 or magnesium chloride ($MgCl_2$) for a

D5. The general approach is to displace unused [γ-^{32}P]ATP, labeled normal nucleotides and other contaminants from the spotting origin onto a Whatman paper wick attached to the layer during D1, while retaining all hydrophobic adducts at or close to the origin. D3 runs in the opposite direction to D1 using a solution of lithium formate and high molarity urea, pH 3.5 (LFU), to displace and separate adducts. D4 runs perpendicular to D3 and further resolves the adducts in a solution of lithium chloride, Tris and high molarity urea, pH 8.0 (LTU). D5 proceeds in the D4 direction using a 1.7 M NaH_2PO_4 solution to remove any remaining radioactive contaminants (a final 'clean-up' step). TLC plates can also be excised at their periphery for decreased background (79,81,132).

Adduct losses result when inappropriate solvents (or solvent concentrations) are used. D1 can be run as described (opposite to D3) or in the same direction in cases where certain adducts migrate several cm away from the origin in D1. An unknown adduct of B[*a*]P (a relatively polar compound) migrates when D1 = 1.0 M NaH_2PO_4 and is lost in this solution if D1 is opposite to D3. Increasing the molarity of D1 to 1.7 M (NaH_2PO_4) will enable retention of this adduct and allow D1 to be run opposite to D3. Adducts with decreased lipophilicity have been shown to migrate in 1 M NaH_2PO_4 (133; A.Garg and R.C.Gupta, unpublished data). In general, we use D1 opposite D3 in 1.0 M NaH_2PO_4, pH 6.0, for known adducts not migrating in D1 and 1.7 or 2.3 M for unknowns or adducts that migrate in D1. Solutions for D3 vary with respect to both concentration of lithium formate and urea; urea content having the greatest effect on adduct migration. Lithium formate has been reported to vary between 1.8 and 8.0 M (117,129,134,135) with a pH of ~3.5. Urea is used at concentrations of 2.0–8.5 M (107,135,136). Concentrations of 7–8.5 M have been shown to be optimal for promoting the migration and resolution of aromatic adducts (two or more benzene rings) during TLC (79). However, in cases of less lipophilic adduct (e.g. <2 benzene rings) high molarity urea solutions are unnecessary and can result in overmigration/loss of adducts in D3. In these situations, lower urea concentration should be used as has been reported for compounds like safrole (134,137) and acenaphthylene.

D4 runs perpendicular to D3 and is usually in LTU. Lithium chloride concentration varies from 0.35 to 1.5 M (125,129, 134,137), Tris 0.2–0.5 (129,134) and urea 2–8.5 M (129,134; and A.C.Beach, unpublished results). Urea concentration is again the important variable as it promotes the further migration of aromatic adducts. The use of commercial (Machery-Nagel) versus laboratory prepared PEI–cellulose layers requires higher (20–25%) concentrations of electrolytes in both D3 and D4 than described in the original procedure (79). In addition, adduct mobility has been reported to vary significantly when using different brands of commercially available sheets (102) as well as within a single batch of commercial sheets.

The use of isopropanol/4 N ammonium hydroxide for D4 at various ratios (v/v) has also been reported (61,62,115). Most crucial is the relative proportion of ammonium hydroxide as high concentrations neutralize the anion-exchange capacity of PEI–cellulose layers and allow for increased migration of highly hydrophobic adducts. The use of this solvent provides, in general, a much clearer resolution of adducts and decreased retention of background versus LTU. Ratios of 0.8–2:1 isopropanol/ammonia have been successfully employed in our laboratory in the migration of adducts as lipophilic as 6-bromomethylbenzo[*a*]pyrene and as small as acenaphthylene.

D5, usually 1.7 M NaH_2PO_4, may be run to enhance the removal of residual radioactive contaminants. In general, the lower the concentration of NaH_2PO_4 the cleaner the map. However, in cases where adducts migrate, solutions of higher molarity need to be used.

D3 and D4 are the most crucial steps in the chromatography of adducts as they are the steps where adducts are actually separated and resolved, and thus deserve further comment. LFU solvent is standard in most postlabeling laboratories. Successful use of D3 depends on the properties of the adducts being analyzed and appropriate choice of urea strength. We use, in general, 7 M urea, and have been able to recover a wide spectrum of hydrophobic adducts of diverse chemical nature. However, in unknown cases some pilot studies are necessary to optimize chromatography prior to full-scale sample screening. D4 is, as mentioned, typically LTU. The use of LFU/LTU is excellent in cases of unknown adducts as most adducts are retained on the chromatogram. These solvents have been utilized in almost all human studies where multiple aromatic adducts of broad range chemical properties are likely.

Diagonal radioactive zones (DRZs) result when many adducts of widely varying polarity are subjected to LFU/LTU chromatography and are only partially resolved (135). In general, DRZs are absent or less extensive in control versus exposed animals and can be single or multiple and compact (139) or highly diffuse (140). Spots may be visible within the DRZ (139) or completely buried (140). Quantitation of exposure based on total radioactivity in the DRZ is questionable based on the high background that tends to be retained. In addition, confounding background spots are retained (described in next section) when using LFU/LTU (141). If adducts related to known compounds (e.g. B[*a*]P) are present, they should be resolved for accurate quantitation. High molarity urea solutions may potentially lead to overmigration of some of the adducts present within the DRZ. Chromatography systems have been developed to allow clear resolution of aromatic adducts with little retention of background adducts or radioactive contamination (62,115,142).

A variety of systems have been developed that complement

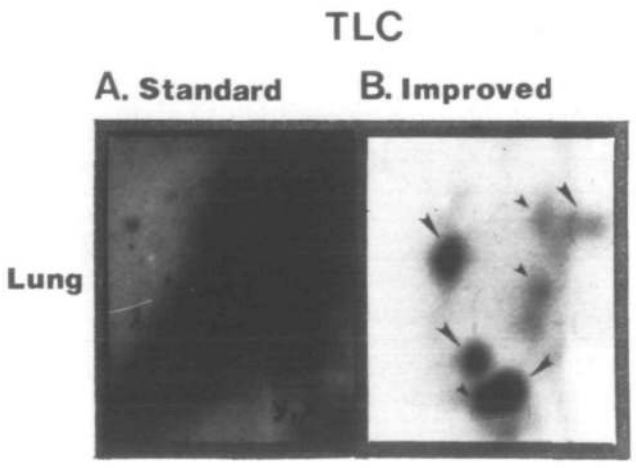

Fig. 7. Comparison of 'standard' 3.5 M lithium formate, 7 M urea, pH 3.5 (LFU)/0.8 M lithium chloride, 0.5 M Tris–HCl, 7 M urea, pH 8.0 (LTU) (D3/D4) chromatography (**A**) and improved LFU/isopropanol–4 N ammonium hydroxide (D3/D4) chromatography (**B**) for resolution of adducts related to mainstream cigarette smoke exposure in rat lung. The diagonal radioactive zone (DRZ) generated in the standard method is poorly resolved and contains test-tube related spots *x* and *y*. Several clearly resolved adducts are present in (B) and *x* and *y* are absent. For details consult ref. 62.

LTU (132). Relative migration in LTU provides an indication regarding adduct properties, which can then be exploited for better resolution in the other solvents. The adducts of compounds like B[*a*]P and 7,12-dimethylbenz[*a*]anthracene (103,105), and aceanthrylene (143), etc., are resolved much better in isopropanol/ammonia versus LTU. Isopropanol/ammonia has in fact been demonstrated to resolve artificial mixtures of many adducts of diverse chemical nature including B[*a*]P, benz[*a*]anthracene, chrysene, benzo[*k*]fluoranthene, dibenz[*a*,*h*]anthracene, benzidine, 4-aminobiphenyl, etc., onto a single map with a very high degree of resolution (62). This solvent system has also resulted in excellent resolution of many adducts detected in the oral mucosal DNA of tobacco users and non-users as well as in the lung DNA of rats exposed to mainstrain cigarette smoke which otherwise migrate in the form of a DRZ (Figure 7) (62,115). For as-yet undetermined reasons, a significant part of the chromatographic background radioactivity is retained at the D3 origin and/or migrates in discrete fronts, thus resulting in usually cleaner maps than with LTU after subsequent D5. Adducts derived from a complex mixture may co-migrate with reference adducts (e.g. BPDE-dG) in the LFU/LTU systems even if they are not related. Any time an unknown adduct is assayed its identity must be confirmed by co-chromatography in multiple solvents (62).

When attempting to assay unknown human samples for adducts, the LFU/LTU approach is still important and suggested in pilot studies focused on optimizing the recovery and separation of adducts. However, once the chromatographic properties of the adducts are known, better resolution is provided by the isopropanol/ammonia solvent system.

High-performance liquid chromatography. HPLC has also been used for the resolution of butanol-enriched adducts following ^{32}P-postlabeling (144). Adducts of 2-aminofluorene, 4-aminobiphenyl, β-naphthylamine, and the alkenylbenzenes, safrole and methyleugenol, have been detected (144). Dual HPLC/nuclease P_1 enrichment coupled with HPLC resolution has also been used for adducts of fluoranthene (145).

The detection of cyclic, low mol. wt adducts using HPLC separation in combination with ^{32}P-postlabeling has also been reported (146,147). Chloracetaldehyde, vinyl chloride, etc., form 'etheno' cyclic nucleic acid adducts such as 1,N^6-ethenodeoxyadenosine and 3,N^4-ethenodeoxycytidine. Chloracetaldehyde-modified DNA enzymatically digested to the 3′-mononucleotide level was separated by ion-pair reverse-phase HPLC. The retention times of adduct standards were used to elute the fractions containing the cyclic adducts which were then postlabeled, 3′-dephosphorylated by nuclease P_1, resolved by HPLC and quantified by liquid scintillation (146). 1,N^2-Propanodeoxyguanine induced *in vivo* by crotonaldehyde or *N*-nitrosopyrolidine was also detected by ^{32}P-postlabeling after HPLC separation using the retention times of the standard adduct (147).

Alkylguanine adducts with open ring, platinated crosslinked guanines and styrene oxide–guanine adducts have also been detected by selective HPLC purification followed by post-labeling and TLC (148,149). N^7-Methylguanine adducts formed *in vitro* or from human samples have been separated with HPLC prior to ^{32}P-labeling with detection limits of 1 per 10^7 nucleotides (150).

Labeled bisphosphates of several PAH diolepoxide adducts have also been resolved by HPLC using a modified silica gel column and a gradient of methanol in a phosphate buffer at low pH and high ionic strength in an attempt to increase the separation/resolution of adducts with similar chromatographic characteristics (151).

Autoradiography. Autoradiography is by far the most sensitive means for detecting labeled adducts. As little as 2–4 c.p.m. can be detected using a 72 h exposure owing to the high specific activity of β-emitting [γ-^{32}P]ATP (152). Du Pont Cronex 4 or the more sensitive Kodak XAR-5 films are used for autoradiography performed in X-ray film cassettes with intensifying screen-enhancement. Film development is either manual or in an automatic film processor.

Exposure is either at room temperature or in most cases at −80°C for marked increases in sensitivity. Exposure time varies from 0.5 to 4 h for adducts ≥1 per 10^4 nucleotides or up to 1–3 days for low adduct levels (1 per 10^7–10^{10}) (132). Adduct maps are typically dotted with either ^{14}C (long exposure, ≥8 h) or ^{99}Tc (short exposure, few minutes to several hours) at their periphery so that they may be aligned properly for locating labeled compounds on the chromatogram.

Step 5: Cerenkov/scintillation counting

Following autoradiography, chromatograms and autoradiograms are aligned on a light box. Individual spots and a blank spot are marked with pencil on the plastic side of the PEI layer. Spots are excised from the chromatogram and transferred (layer side down) to wide mouth (22 mm i.d.) plastic scintillation vials. Ethanol (2 ml) is added to prevent flipping of the cutout which may affect counting efficiency. Radioactivity is assayed by either Cerenkov counting (i.e. without the addition of scintillation fluid) or after the addition of scintillation fluid. Proper alignment and use of blank spots equal in size to adduct spots is necessary to obtain true count rates (79).

Adduct quantitation procedures have been previously described (132). Determination of adduct levels is via relative adduct labeling (RAL) where RAL = c.p.m. in adducts/c.p.m. in total nucleotides × 1/dilution factor.

Limitations and confounding factors

Inherent to any highly sensitive assay are the presence of confounding factors which may lead to false interpretation of results. Frequently encountered factors are briefly summarized here.

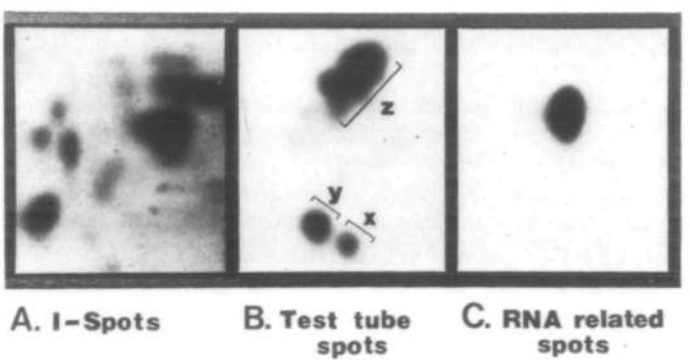

Fig. 8. ^{32}P-Maps of potential confounding factors which can be falsely interpreted as exposure-related adducts. (A) I-spots from the liver of 1 year old rats as analyzed by the nuclease P_1 version of the ^{32}P-postlabeling assay with LFU(propanol/4 N ammonium hydroxide) in D4/D3 (ref. 61). (B) Calf thymus enzymatically hydrolyzed, butanol extracted and ^{32}P-labeled in a conical 1.5 ml polypropylene tube with LFU/LTU (D3/D4) chromatography. (C) RNA from rat liver analyzed under conditions described for (B). For LFU/LTU solvents consult Figure 7 legend.

I-compounds. I-spots/compounds (Figure 8A) is a collective term for the wide array of chromatographically separable indigenous (arising in the absence of exogenous treatments) adducts found in animals/humans that are tissue, species, sex, diet and age specific (153). These spots are known to be almost undetectable in newborn animals but increase with age and are speculated to play a role in carcinogenesis (153). I-spots behave like carcinogen−DNA adducts versus natural modifications like m^5C and dilute in regenerative tissues like the liver (154). In addition, liver I-spots have been shown to be susceptible to modulation in rats given treatment with hepatocarcinogens like 2,3,7,8-tetrachloro-*p*-dibenzodioxin, peroxisome proliferators and carbon tetrachloride (155,156). Treatment with these compounds decreases the age-related increase of I-spots in control animals. Standard rodent chow diets, however, increase the complexity and level (2.5- to 6.4-fold) of I-compounds versus purified diets (157).

I-compounds represent a complex series of several spots of highly varying chromatographic mobility (polarity) which may be misconstrued as carcinogen-induced DNA adducts. Appropriate chromatographic controls are therefore vital for the correct interpretation of these background spots.

Test-tube spots. The presence of 1−3 spots unrelated to carcinogen exposure (Figure 8B) in DNA from any source including humans, rodents and fish, has been described previously (158,159). These spots are most prominent following enrichment with butanol or HPLC (*x* and *y*) and to a lesser extent following nuclease P_1 (*y* is nearly absent), suggesting partial lability in the presence of nuclease P_1 (85,87,88,160; and R.C.Gupta, unpublished data).

Several groups have suggested the spots to be normal DNA constituents (I-compounds) or possible artefacts of DNA digests (106,141). Similar spots have been shown to form spontaneously when an aqueous solution of calf thymus DNA is incubated in plastic (polypropylene) test-tubes at room temperature (161). Several studies in our laboratory have shown that these spots (*x*,*y*,*z*) are related to these plastic tubes. The intensity of these spots varies depending on the source of plastic tube and the temperature and length of time to which DNA/nucleotide samples are exposed during the DNA isolation, digestion, enrichment and labeling. Unidentified chemicals appear to leach from the tube and react with DNA to form adducts with guanine, adenine and cytosine, which are detected by postlabeling when using the LFU/LTU chromatography system (R.C.Gupta, manuscript in preparation). If the isopropanol/ammonia system is used such spots move out of the chromatogram.

RNA-related spots. RNA, unlike DNA, contains many natural and structurally diverse modified bases. A particular hyper-modified tRNA base, chromatographically identical to 2-methyl-thio-N^6-isopentyladenosine (Figure 8C) was found to be present in tissues of rats and humans and was readily detectable by the butanol version of the ^{32}P-postlabeling assay only (162). As-yet unidentified, modified components have been detected only when using the nuclease P_1 version of the assay (R.C.Gupta, unpublished data). The presence of naturally modified bases in RNA which are substrates for ^{32}P-postlabeling can also lead to erroneous results if RNA-free DNA is not prepared.

Mention of these 'system-spots' is made here so that they should not be interpreted as carcinogen-induced adducts. In cases where good controls are not available, such as with human studies, animal DNA controls as well as DNA blank controls (minus DNA) should be assayed in parallel to ensure accurate interpretation of data.

Tetrols as substrates for PNK labeling. A lack of complete substrate specificity of PNK has also been reported. Tetrols of B[*a*]P and chrysene have been shown to be substrates for PNK and are labeled by [γ-^{32}P]ATP in the absence of nucleic acids (163). However, the labeling efficiency of the B[*a*]P tetrol was 2000-fold less versus BPDE−DNA adducts. Parent hydrocarbons, phenols, dihydrodiols and triols were not suitable substrates for kinase labeling (163). This work has been confirmed by others and it would seem that the labeling of the non-adduct substrates may occur in cases where DNA adducted *in vitro* is insufficiently purified (102). Residual tetrols have been shown to remain in *in vitro* generated samples even following extraction with ether or ethyl acetate (163). Such contaminants are, however, not expected to lead to false positives from *in vivo* samples as DNA isolated from animal and human tissues is purified by a series of solvent extractions and precipitation with ethanol.

Other potentially confounding factors. In addition to the factors described above, others may also affect interpretation of data. Poor chromatography can result in incomplete separation of adducts, smears of radioactive contaminants, etc., which can complicate interpretation of adduct maps. Removal of urea and/or salt from the chromatograms by washing with water at each step of the multidirectional TLC is also important to generate clean maps. Contaminated X-ray cassettes can produce false adduct images on films used for autoradiography. Cross-contamination when handling samples must also be avoided. Highly adducted (1 adduct per 10^2-10^5 nucleotides) reference DNAs should be processed separately or with extreme care while analyzing unknown human DNAs. Stainless-steel needles have been found to adsorb lipophilic adducts and unless washed with appropriate solvents can easily cross-contaminate samples. We suggest, if possible, the exclusive use of disposable tubes, Pasteur pipets, and pipet tips to eliminate the introduction of even minor amounts of contaminants between samples.

Other limitations

Several investigators have reported a lack of quantitation of human DNA adducts using ^{32}P-postlabeling. Comparisons with other adduct detection methods often provide conflicting results. This is especially true with human samples where exposure is unknown, and thus antibody cross-reactivity or the presence of many other similar fluorophores can lead to overestimation of binding. The most reasonable way truly to quantify adducts produced by a given chemical is to synthesize a radioactive analog for comparative prelabeling and postlabeling binding studies. This has been done in a limited number of studies and binding detected by ^{32}P-postlabeling was similar (60−90%) to binding produced by radiolabeled carcinogens (164,165). This is an important area deserving further evaluation.

Human studies with ^{32}P-postlabeling

Approximately 50 studies related strictly to ^{32}P-postlabeling adduct analysis of human tissues from biopsies, autopsies and phlebotomy for the purpose of measuring exposure-related effects have been published to date—many in 1990−1991 alone. Discussed below, and summarized in Table II, are studies related to cigarette smoking, occupational and clinical exposure, and a group of investigations related to human biomonitoring from

Table II. DNA adduct data compiled from ^{32}P-postlabeling analysis of various human tissues and cells

Exposure/tissue	Enhancement procedure	No./nature of adducts[a]	Adduct levels[b] adducts/10^9 nucleotides	Ref.
Cigarette smokers				
Lung	P_1	DRZ[c]	6–340	60,167
Lung/heart	P_1	DRZ	10–250	88,106,109
Lung	HPLC	O^6-methyl-dG	100–5200	150,191
Lung	HPLC	O^6-ethyl-dG	200–3600	191
Lung	HPLC	N^7-methyl-dG	1400–7200	150
Lung	HPLC	dG-C8-ABP	10–850	191
Lung	P_1	DRZ/dG-N^2-B[*a*]P[d]	19–340[d]	169
Lung	P_1	DRZ	14–134	119,140
Lung/heart	P_1/butanol	2–6	≤100	201
Lung	ATP-deficient	4–7	170–290	166
Bronchoalveolar lavagate	P_1/butanol	2–4	≤200	202
Placenta	ATP-deficient	1–4	5–12	173,184
Placenta	P_1/butanol	dG-N^2-B[*a*]P	≤200[e]	102
Bladder	P_1/butanol	6–9	≤50	116
Bladder	P_1/butanol	up to 5	13–270	171
Urothelial cells	P_1/butanol	2–6	1–25	172
Urinary mutagens	P_1/butanol	up to 7[f]	not applicable[g]	117,203
PBL	P_1	1–6	≤10	108
WBC	P_1	DRZ	20–25	119
WBC	P_1	4	very low[h]	190
PBL	P_1	10	100–600	176
PBL	butanol	2–5	≤30	204
Monocytes	P_1	2	not reported	107
Tobacco chewers/cigarette smokers				
Oral mucosa	ATP-deficient	up to 5	1–100	177
Oral mucosa	butanol	up to 16	5–200	115
Oral mucosa	butanol	DRZ	up to 1600	178
Occupationally exposed				
WBC (foundry)	P_1	up to 15	2–200	111,179
WBC (foundry)	P_1	DRZ	90–260	76
WBC (coke oven)	P_1	DRZ	27–530	77
WBC (roofers)	P_1	2–3	2–130	114,125
WBC (styrene)	none	2	2200	185
Clinically exposed				
Skin(coal/juniper tar)	P_1	DRZ	2–150	112,113
Liver (mitomycin C)	P_1	up to 4	6–30	123
WBC (mitomycin C)	P_1	up to 4	≤10	123
WBC (procarbazine/dacarbazine)	HPLC	N^7-methyl-dG	250–5700	186
Undefined exposure				
Bone marrow	ATP-deficient	5	10–90	205
Gastric/duodenal	P_1	DRZ	1–40/1–109	193
Colon	P_1	1–2	10	160
Mammary	P_1	1–2	4–30	192

[a]Except where indicated, the only class of compounds analyzed were aromatic/lipophilic adducts, i.e. class 4 adducts according to our classification scheme (see Table III below).
[b]Levels where reported in femto or attomol/μg DNA were converted to adducts/10^9 nucleotides considering 1 μg DNA = 3 × 10^9amol.
[c]DRZ = diagonal radioactive zone of unresolved radioactive compounds.
[d]Part (2–42 adducts/10^9 nucleotides) of the adduct radioactivity was attributed to the presence of dG-N^2-B[*a*]P.
[e]Estimated from data of known standards.
[f]PhIP (see Table I) identified as the major metabolite.
[g]It seems difficult to correlate levels of DNA-reactive metabolites (DRM) to tissue DNA adducts.
[h]Actual levels not reported, probably in the range of 10–30 adducts/10^9 nucleotides.

undefined exposures. For details, methodological considerations or adduct levels, the reader is referred to Table II or the cited references.

Cigarette smoking

Cigarette smoke is known to contain 3000+ chemicals, at least 50 of which have been identified as mutagenic/carcinogenic (56).

Table III. ^{32}P-Postlabeling classification scheme for adducts arising from a diverse spectrum of chemicals[a]

Class	Chemical carcinogen	^{32}P-Postlabeling conditions				
		Enrichment[b]			TLC solvents[f]	
		Butanol	Nuclease P_1	Others	1st dir.	2nd dir.
1	dimethylnitrosamine	−	−	+[c]	A (40%)[g]	B
	ethylnitrosourea	−	−	−	A	B
2	diaziquone	−	+	+[d]	A (20%)	C
	acrolein	−	−	+[c]	D (50%)	E (50%)[h]
3	styrene	−	+	+[d]	A	C
	safrole	+	+	−	D (60%)	E (60%)
	IQ	−	−	+[e]	D (50%)	E (60%)
4	4-aminobiphenyl	+	−	+[c,d]	D	E
	benz[*a*]anthracene	+	+	+[c,d]	D	E
	benzo[*a*]pyrene	+	+	+[c,d]	D	E
5	6-methylbenzo[*a*]pyrene	+	+	+[c,d]	D[i]	E[i]
	7-methylbenz[*a*]anthracene	+	+	−	D[i]	E[i]

[a]Based on reported PEI−cellulose TLC behavior, a tentative scheme is developed for adduct separation by class. 'Small' adducts (methylated, ethylated, etc.) are grouped as class 1. Adducts with an increasing degree of lipophilicity are classifed as class 2−5. The classification is based on retention of labeled adducts on C_{18} reverse-phase (class 2−5) or PEI−cellulose layer (class 4 and 5) in combination with requirement of plain electrolyte solution for adduct separation (class 2) or electrolyte solution with increasing concentration of urea to 3−5 M (class 3) or 7−8.5 M (class 4). Class 5 is reserved for extremely lipophilic compounds which remain at or close to the origin despite the presence of high concentrations of urea. The scheme delineates adduct separation by class for most or all chemicals that are expected to be present in the human environment.

[b]The most commonly used butanol and nuclease P_1 procedures are presented separately, while alternative procedures are grouped as 'others'. '+' represents extensive adduct recovery; '−' denotes slight or no recovery ('−' appearing under other procedures signifies alternative procedures as yet unexplored).

[c]HPLC enrichment.

[d]C_{18} TLC enrichment (G.G.Spencer and R.C.Gupta, unpublished results).

[e]ATP-deficient enhancement procedure.

[f]TLC solvents. Class 1 adducts are separated in the presence of vast excess of labeled normal nucleotides and other contaminants. Class 2/3 adducts (by C_{18} TLC) and class 4/5 adducts (by PEI−cellulose TLC) are pre-purified (i.e. removal of residual normal nucleotides and other contaminants) and then separated. The commonly used solvents are: A = 2.0 M ammonium formate, pH 3.5; B = 0.4 M ammonium sulfate; C = 3.0 M sodium phosphate, pH 5.0; D = 3.5 M lithium formate/7 M urea, pH 3.5 (LFU); E = 0.8 M lithium chloride/0.5 M Tris−HCl/7 M urea, pH 8.0 (LTU).

[g,h]Prepared by dilution with water of the full-strength solvents.

[i]Adducts remain at or close to the chromatogram origin in standard solvents E and/or F. Alternative solvents are needed for separating adducts (K.Stansbury, M.Chacko and R.C.Gupta, unpublished results).

Several investigators have studied the formation of DNA adducts in various human tissues in relation to cigarette smoking.

Lung and associated tissues/cells. The presence of DRZs of adducts was found to be dose- and time-dependent in the DNA from the non-tumorous bronchial and larynx tissues of autopsied or biopsied smokers which were absent or present to a much lesser extent in non-smokers (60,109,119,166−168). Former smokers who had stopped smoking only 3 months prior to surgery had levels typical of current smokers, whereas those who had ceased consumption for at least 5 (in some cases up to 14) years prior to the study had levels similar to the non-smokers (60,109). Autopsy samples have also revealed the presence of smoke-related DNA damage in other organs. Binding was most extensive in the lung and heart but was also found in the kidney, bladder, esophagus, liver and ascending aorta (109).

Adduct level in bronchial tissue was shown to be similar in all lobes of the lung as well as between alveolar and bronchial or tumorous and non-tumorous tissue from the same individual (88). Bronchial adducts were strongly associated with cigarette consumption and were potentiated by the frequent intake of alcohol (88). Patients smoking non-filtered cigarettes had greater levels compared with those smoking filtered brands (169). A spot co-chromatographing with BPDE-dG has also been shown in the DRZs of smokers (169).

DNA binding has been compared with the induction of aryl hydrocarbon hydroxylase (AHH) in the non-neoplastic lung parenchyma of lung cancer patients (140,170). Levels of both adducts and AHH were higher in smokers as compared to non-smokers.

Bladder. Biopsy and autopsy samples of human bladder have also been analyzed for DNA adducts, and support a relationship to cigarette consumption (116,167). Adducts similar to those reported previously in lung and placenta were found with one spot co-migrating with a 4-aminobiphenyl reference standard (116). Recent analysis of 73 human bladder biopsies, however, suggested no significant differences between these groups and was thus in disagreement with the previously mentioned studies (171).

Exfoliated urothelial cells from 40 patients have also been examined for smoking-related adducts (172). Four adducts appeared to be related to smoking as they were 2−20 times higher in smokers versus non-smokers and one co-migrated with *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (172). Total DNA binding was, however, not significantly different between smokers and non-smokers.

Placenta. Several modified nucleotides, including one strongly associated with maternal smoking, have been found in term placentas (173,174). Three adducts were shown to be nearly exclusive to maternal cigarette smoke exposure and the levels were proportional to maternal consumption of cigarettes and inversely related to birth weight (175).

The presence of BPDE-dG in the placental DNA of smoking and non-smoking mothers has also been reported (102). An adduct that appeared to have similar chromatographic properties as a highly adducted BPDE-dG standard was detected using either the nuclease P_1 or butanol-enrichment versions of the assay (102).

White blood cells. Limited differences between DNA adduct levels in smokers and non-smokers have been reported in total WBCs (119,168) or PBLs (108), indicating the potential limitation for use of WBCs as a surrogate tissue. In another study, however, when DNA isolated from granulocytes and lymphocytes of smokers and non-smokers was analyzed, binding in lymphocytes was significantly higher in smokers versus non-smokers, while binding in granulocytes was not significantly different between the two exposure groups, suggesting the importance of separation of cell types for analysis (176).

Male volunteers subjected to mainstream and passive smoke were tested post-exposure for adducts in their blood monocytes (107). Qualitatively similar adducts were present in all individuals prior to the study regardless of smoking status. Additional, though short-lived, adducts appeared soon after smoke exposure in the monocytes of previously active smokers. Adducts related to exposure did not appear in the exposed, but previous non-smokers (107).

Tobacco chewing/snuff dipping

The exfoliated oral mucosal cells of populations at risk for oral cancer (Indian betel-nut and Khaini chewers and Filipino inverted smokers) were compared with Canadian non-smoking controls (177). Chromatographically distinct adducts of unknown origin were found in all individuals; however, no adduct was related to exposure (177).

Similar results were obtained from the oral mucosa of tobacco chewers from Bombay, comparable controls from within the same community, and a collection of random smoking and non-smoking controls from Houston, Texas (115). However, two adducts were lost during nuclease P_1 enrichment, suggesting their potential aromatic amine origin (115).

The effects of smoking and alcohol consumption on adduct formation in oral mucosa has also been investigated (178). There were no adducts consistently related to either smoking or alcohol intake; however, total binding in smokers was higher versus non-smokers.

Occupational exposure

^{32}P-Postlabeling has also been applied to assess occupational exposure to PAHs. Iron foundry workers, coke-oven workers and roofers comprise the main populations of study.

Foundry workers. Epidemiological data have suggested that increased exposure to airborne PAHs contributes to increased risk of lung cancer in this population. Airborne levels of B[*a*]P were used to estimate exposure (111). Exposure-dependent adducts were detected in WBCs of foundry workers, with no additional effects related to smoking, age or gender being observed (76,111,179,180). However, large interindividual variations, characteristic of human studies, existed. All adducts were chromatographically distinct from BPDE-dG (111). PBLs as opposed to total WBCs have been found to contain a number of adduct spots in exposed individuals versus controls and at levels nearly 50-fold higher (110).

Coke-oven workers. Coke ovens are known to emit PAHs in relatively large amounts into the environment. WBC-DNA of workers from coke-oven plants in Poland, local controls and countryside controls has been analyzed for the presence of exposure-related aromatic adducts (181–183). Binding was qualitatively and quantitatively similar in workers and local controls, but was considerably lower in rural controls (181). Neither smoking nor occupation of the local controls could explain the adduct similarities to plant workers, and thus environmental pollution via the coke-oven plant has been implicated. Norwegian coke-oven plant workers (90%) also had detectable aromatic adducts in their WBC-DNA (184).

Roofers. Epidemiological associations have been made between the risk for skin and lung cancer and exposure to PAHs in hot coal-tar pitch via roofing (114). DNA adducts were detected in 10 of 12 roofers (114,125). Adducts did not co-migrate with BPDE-dG and cigarette smoking did not appear to be a factor. Skin-wipe tests measuring the levels of several PAHs post-workshift correlated well with adduct levels suggesting the role of dermal absorption (114).

Styrene. Two major adducts, which appeared to co-migrate with adducts formed *in vitro* by interaction of mutagenic styrene 7,8-oxide and DNA, were detected in the lymphocyte DNA from a single worker exposed to an estimated 47 p.p.m. styrene/8 h shift (185). No correlations have yet been made between exposure and adduct levels.

Clinical exposure

Chemotherapeutic agents with DNA binding capacity have been implicated in the induction of secondary malignancies in patients treated with these chemicals. In addition, agents used for the treatment of non-cancer-related diseases such as coal- and juniper-tars for the treatment of psoriasis are suggested to be potential carcinogens.

Chemotherapeutic agents

Mitomycin C, given for the preoperative regression of hepatocellular carcinoma, has been shown to induce four adducts in the liver and WBCs of patients treated with this drug (123). Adducts in WBCs were qualitatively identical to those in the liver but 2–3 times lower.

7-Methylguanine residues were detected in the WBC-DNA of cancer patients treated with the chemotherapeutic agents procarbazine and dacarbazine (186).

Coal- and juniper-tar for psoriasis. These agents, by virtue of their high PAH content, have been implicated to increase the risk for skin carcinoma in patients receiving them for psoriasis therapy (187,188). Single doses of coal-tar, even pharmaceutical grade, are carcinogenic in animal skin tumorigenicity models (113).

Skin biopsies of patients treated once a day for 5 days and sampled 24 h post-treatment contained a pattern of aromatic DNA adducts (112,113,189). Animal adduct patterns and levels were similar to humans; however, these agents were found to interact with distal organs including the lung (typical PAH target) (113).

Others

Several investigations falling into categories not fitting the above designations are mentioned here.

Residential wood combustion smoke (RWC). The increased use of wood as a heating source has elicited concern regarding household exposure to carcinogenic PAH emissions (190). Placental and WBC-DNA from exposed and unexposed women was analyzed for the presence of aromatic adducts related to RWC. All placental specimens contained one major (47 ± 10% of the total radioactivity) and 12 minor spots not found in WBC-DNA. Four spots not detected in placental DNA were present on the maps from WBCs. There were no adducts related to RWC, nor did any spot co-migrate with BPDE-dG (119).

Lung tissue. Lung autopsy specimens have also been examined for the presence of alkyldeoxyguanosine adducts using an HPLC-enrichment method prior to labeling and adduct resolution (150, 191). O^6-Methylguanine, O^6-ethylguanine and *N*7-methyl-

guanine have been detected, indicating exposure to unspecified alkylating agents (150,191).

Mammary epithelial cells. DNA adducts were found in the mammary epithelial cells from 3/10 donors and exhibited distinct chromatographic patterns unrelated to BPDE-dG (192). The storage potential of fatty breast tissue, the metabolic capability of mammary cells and the prevalence of breast cancer necessitates detailed biomonitoring studies aimed at this cell type.

Gastrointestinal tissue. DNA adducts were measured in duodenal and gastric tissues of patients with familial adenomatous polyposis (FAP) (193). Ninety per cent of all patients were found to possess aromatic DNA modifications. Binding in FAP duodenal tissue was greater versus duodenal tissue from controls and gastric was much less versus duodenal from the same patient (193). This evidence suggests the increase in adducts found in the duodenal tissue of FAP patients may be related to the increased incidence of neoplasia in this tissue. No effects related to age, gender or smoking status were present.

Comments

The ^{32}P-postlabeling assay has been applied almost exclusively to the detection of bulky aromatic adducts (class 4, see Table III below) formed presumably following exposure to polyaromatic-rich complex mixtures. However, many other genotoxic chemicals are present in these mixtures which are likely missed by standard postlabeling protocols. Only recently have a limited number of studies been published that deal with the detection of smaller/less lipophilic adducts (classes 1–3, see below). In the analysis of human lung, bladder and WBC/PBL-DNA from tobacco smokers only a few resolvable adducts appeared to be related to smoking. The oral mucosa of individuals who smoke or chew tobacco was devoid of DNA adducts related to tobacco exposure. However, both populations (smokers/chewers) are clearly at elevated risk for development of cancers related to their habits. A potential reason for the lack of correlation between exposure and the formation of adducts in these populations is that the methodological approaches taken detect only one class (class 4, see below) of adduct. Adducts related to less lipophilic compounds prevalent in tobacco such as 4-[methylnitrosamino-1(3-pyridyl)]-1-butanone (NNK) or toluidine are missed possibly due to lability during enrichment or overmigration during TLC. A few comparison studies have reported similar findings in the lung DNA of smokers enhanced with either butanol or nuclease P_1; however, adducts in human bladder and oral mucosa have been reported to be lost during nuclease P_1. The highly variable nature of human samples should preclude the exclusive use of only one enhancement or TLC procedure.

With limited modifications, chromatographic conditions originally described in 1982 (79) are used in the majority of human studies resulting in bands of incompletely resolved aromatic adducts and background contamination called DRZs. DRZs, though providing a simple means to visualize exposed versus unexposed individuals, are subject to false interpretation and potentially result in the loss of highly important information. DRZs vary from laboratory to laboratory as either compact, semi-resolved bands of adducts to completely diffused smears of background contaminants with no visible adducts. Distinct adduct spots, if present, may be completely buried in the DRZ, and thus no information regarding their R_f or potential chromatographic resemblance to known adducts may be obtained. Test-tube related adducts (Figure 8B) are also present and often hidden within DRZs where they may be falsely incorporated into the quantitation of total radioactivity within the DRZ, particularly if the butanol or HPLC-enhancement versions of the assay were used. The level of these spots, notably *x* and *y*, varies greatly, potentially resulting in overestimation of exposure. In addition, quantitative analysis of exposure based on DRZs is subject to some criticism as no standardized method exists.

In general, the limited findings in DNA from human sources may not be due to absence of exposure-related adducts *per se*, but due to the use of conditions that may miss those adducts which are present. In analyzing unknowns, an approach targeting more than one class of adducts (see below) is vital, and thus variable enrichment and TLC conditions need to be chosen that allow detection of a broad spectrum of chemicals binding to DNA. Caution and scrutiny must be exercised when following published protocols that may not be suitable for a specific purpose.

Interlaboratory discrepancy. Several tissues have been analyzed by the ^{32}P-postlabeling assay in a number of independent laboratories for the presence of adducts related to cigarette smoking, occupational or clinical exposures, etc (Table II). In many cases, independent investigators have reported similar findings. However, interlaboratory variability may occur due to differences in tissue sources, tissue handling or technical approaches during the course of the postlabeling assay.

To address briefly the point of tissue source/handling, tissues obtained from autopsy may be questionable with regard to adduct integrity. Adducts may not withstand autolytic processes occurring post-mortem, and thus the time-frame of tissue procurement could potentially be vital. Even with biopsy samples, proper care needs to be taken to store the samples immediately at low (−70 to −80°C) temperature to avoid possible adduct losses. In addition, the stability of adducts during prolonged storage (even at −80°C) prior to analysis is not completely known. Certain (PAH) adducts are more stable during storage than others (such as arylamines). In general, prolonged storage of DNA should be avoided especially with unknown human samples.

Methodological practices may also contribute to interlaboratory variation. The analysis of WBC-DNA isolated from the same iron-foundry workers (described above) was carried out independently in three separate laboratories (76). Data were variable both qualitatively (with respect to adduct maps) and quantitatively (2- to 3-fold variations in adducts with the same samples). Interlaboratory discrepancies were likely due to methodological variations made during each phase of the assay. Two laboratories digested their DNA with MN/SPD for 20 h versus 3.5 h in the third laboratory, the nuclease P_1 substrate ratio was up to 2-fold different, the amount of [γ-^{32}P]ATP used for labeling varied from 30 to 200 μCi, and the source of TLC layers as well as the chromatography conditions were highly variable (76). Correlations between the laboratories ranged from $r = 0.45$ to $r = 0.62$ and are likely to be largely due to the lack of similarity in postlabeling methodology.

Similar interlaboratory discrepancies have been shown for the WBC-DNA of coke-oven workers examined by independent laboratories (182). Although a similar trend in data was shown, binding varied 2-fold and correlation between the laboratories was only $r = 0.66$. Variable technical approaches between these laboratories could account for this discrepancy.

Other methodological factors (discussed above) that could contribute to discrepant findings between independent laboratories include: (i) irregular protocol with respect to E:S ratios, pH and

duration of MN/SPD and nuclease P_1 incubations; (ii) heating of samples to 95°C following MN/SPD and/or nuclease P_1 to end enzymatic reactions prior to labeling, a process that could depurinate adducts affecting quantitation; (iii) parallel processing of highly adducted reference DNAs with human samples resulting in cross-contamination of human samples and potential false positives; (iv) co-chromatography of high-level reference adducts (≥ 1 per 10^2-10^5) with human samples where binding may be 1 per 10^7-10^{10} instead of at a 1:1 ratio; and (v) co-chromatography of reference and unknown adducts in only one solvent system and/or systems giving low R_f (0.1−0.2 versus 0.5−0.6) values, and thus inadequate separation and false identification. An adduct from smoke-exposed animals, which co-migrated with BPDE in certain solvents, was shown to separate from that reference adduct when the R_f was increased to ≥ 0.5 (62).

Interlaboratory comparison between our laboratory and the laboratories of Drs J.Ross and S.Nesnow, have given consistently comparative (±10% variation) results (unpublished data). We feel that this is largely due to the similarity in assay conditions between the two laboratories.

Conclusions, recommendations and future directions

The detection of covalent adducts formed when genotoxic agents interact with DNA has become increasingly important in the assessment of human exposure to carcinogens. Several methodologies, including immunoassays, fluorescence assays and ^{32}P-postlabeling, have been developed over the last 10−12 years which allow for the sensitive detection of adducts. Human biomonitoring, by virtue of its differences to animal bioassays, demands a method that is sensitive enough to detect the effects of subtle, low-level exposures to complex chemical mixtures of unknown composition.

The ^{32}P-postlabeling assay has emerged as the method of choice for the detection and quantitation of carcinogen−DNA adducts. Since its development a decade ago, ≥340 papers have been published using the assay to detect the binding of over 140 chemicals (Table I). Application of the ^{32}P-postlabeling approach to such a diverse group of chemicals or mixtures as listed in Table I speaks for the versatility of the assay and makes it ideally suited for human biomonitoring. Nearly 50% of these articles were published in 1990−1991 alone, and ~1/6 are related to human biomonitoring. The primary and unique features of this assay that have resulted in its widespread applications include the following:

- Sensitivity: 1 adduct per 10^{10} nucleotides (≤ 1 per human genome).
- DNA requirement: 1−10 μg; applicable to human samples that may provide little DNA.
- Versatility:
  - —Prior knowledge of adduct composition is not necessary making it applicable to unknown chemicals including those encountered in daily life.
  - —Ability to detect, simultaneously, adducts of different chemical character such as would be found in complex environmental mixtures.
  - —Detection of adducts resulting from interaction of presumably, but unknown, endogenous electrophiles.

The following are recommendations for analyzing human tissue samples for DNA adducts:

- Preparation of RNA-free DNA.
- Standardized conditions for enzymatic digestion, adduct enrichment and labeling.
- Separation of adducts by class (see Table III).
- Selection of TLC solvents providing resolution of adducts.
- Use of appropriate external standards for comparison.
- Co-chromatography with reference of appropriate adduct levels.
- Customization of assay for specific class of adducts to be analyzed.
- Representation of adduct levels in one common unit—adducts per 10^9 nucleotides—so that data from different laboratories can easily be compared.

The ^{32}P-postlabeling method, in its current form(s), has taken several years to develop; however, the assay has yet to reach its full potential. Several important areas for future development include: (i) use of alternative tissues/body fluids; (ii) detection of 'small' adducts including hydroxylated bases induced by oxidative damage; and (iii) classification and standardization of the assay.

Alternative tissues/body fluids

The presence of DNA-reactive metabolites (DRM) has been shown in the blood serum of B[*a*]P-treated mice (95,194) and urine of smokers in combination with a metabolic activation system (117). Adducts were shown to result when these fluids were reacted *in vitro* with DNA. We have recently expanded the use of blood serum as a potential source of DRM for other chemicals using mouse and rat models (A.Garg, A.C.Beach and R.C.Gupta, submitted). Adducts formed from the *in vitro* reaction of serum from mice or rats exposed *in vivo* to a number of carcinogens are similar to those formed in target and non-target tissues. The $t_{1/2}$ of mouse serum albumin, the putative carrier of many DRM, is 1−2 days while rat varies over 2−3 days. Detection of adducts in these animals at times post-exposure appears to be related to this $t_{1/2}$, and thus the 20−25 day $t_{1/2}$ of human serum albumin could potentially extend the window of detectability. Successful application to humans could obviate the need for DNA-containing tissues—a major obstacle in biomonitoring studies.

Detection of 'small' and 'non-bulky' adducts

Many lines of evidence indicate that DNA lesions produced by alkylating (methylating/ethylating) agents or damage induced by oxygen radicals are far more extensive and potentially more important compared with those formed by polyaromatics. However, >95% of the work done thus far by ^{32}P-postlabeling with human specimens has involved the detection of adducts resulting from the interaction of bulky aromatic compounds with DNA. Present advances using micropreparative C_{18}-HPLC (146−150) and TLC (G.G.Spencer and R.C.Gupta, unpublished work) techniques to purify adducts related to small and non-bulky compounds prior to labeling are becoming successful.

Combined HPLC/^{32}P-postlabeling methods for detecting O^6-alkyldeoxyguanosine as well as N^7-methyldeoxyguanosine have been developed and applied for the analysis of these modified bases in human tissue samples with limits of detection in the 1 adduct per 10^7 nucleotides range (150,186,191,195). Additional ^{32}P-postlabeling methods that have been developed for the detection of 'small' adducts but not as yet applied to human samples include (in addition to those mentioned in a previous section on HPLC) those for detecting thymine glycols (130,

196,197) and 8-hydroxydeoxyguanosine (198,199) which result when DNA is damaged by oxygen radical producing agents.

Development of adduct detection and identification strategies

^{32}P-Postlabeling can be adapted to detect virtually any chemical binding with DNA. Classification of these chemically diverse compounds into manageable groups with defined, group-specific detection approaches may give a foundation of objectives on which standard methodologies may be built. Depicted in Table III is a tentative classification scheme ranging from class 1 (polar → slightly polar) to class 5 (extremely lipophilic). Included in this table are suitable enrichment and chromatography protocols for adduct separation and resolution. We feel that the further development of specific adduct classes with proven detection approaches will lead to better and increasingly standardized application of the assay to the broad spectrum of genotoxic agents that humans encounter.

Characterization of ^{32}P-labeled adducts has thus far depended upon chromatographic resemblance with reference adducts. Selection of suitable candidate adducts is, however, difficult when adducts result from complex mixtures such as cigarette smoke. Ideally, identification of adducts should be based on spectroscopic analysis. The quantity of adducts that can be conveniently isolated from human tissue DNA is generally several orders of magnitude lower than the hundreds of nanograms of pure material for mass spectroscopic analysis even in combination with fast atom bombardment. While spectroscopic techniques continue to improve, alternative procedures need to be developed to gain insight into the chemical nature of unknown adducts resulting from complex mixture exposures. Such procedures may include selective chemical reactions (sensitivity of PAH diolepoxide related adducts to $NaIO_4$ oxidation, preferential depurination and hence loss of dG-C8-arylamine adducts, etc.), nuclease P_1 sensitivity of dG-C8-arylamine adducts (103,104), differential pH-dependent solvent partitioning (200) and chromatographic comparison with suspect candidate adducts in multiple solvent systems.

We feel that further applications of the ^{32}P-postlabeling assay will depend on its enhanced utility as a comprehensive and routine assay for human biomonitoring. Future emphasis needs to be placed not only on the detection of other adduct classes, but also on the structural identification of adducts and subsequent confirmation of the etiological agents responsible for the induction of DNA damage and cancer.

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